



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

A protocol for studying structural dynamics of proteins by quantitative crosslinking mass spectrometry and data-independent acquisition

Citation for published version:

Müller, F & Rappsilber, J 2020, 'A protocol for studying structural dynamics of proteins by quantitative crosslinking mass spectrometry and data-independent acquisition', *Journal of proteomics*, vol. 218, 103721. <https://doi.org/10.1016/j.jprot.2020.103721>

Digital Object Identifier (DOI):

[10.1016/j.jprot.2020.103721](https://doi.org/10.1016/j.jprot.2020.103721)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Journal of proteomics

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



A protocol for studying structural dynamics of proteins by quantitative crosslinking mass spectrometry and data-independent acquisition

Fränze Müller¹ and Juri Rappsilber^{1,2}

¹ Bioanalytics, Institute of Biotechnology, Technische Universität Berlin, 13355 Berlin, Germany

² Wellcome Centre for Cell Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3BF, Scotland, United Kingdom

FM: orcid.org/0000-0003-3764-3547

JR: orcid.org/0000-0001-5999-1310

Abbreviations

ACN - acetonitrile

AGC - automatic gain control

ANOVA - analysis of variance

BS³ - bis[sulfosuccinimidyl] suberate

CL - crosslinking

CLMS - crosslinking/mass spectrometry

CV - coefficient of variation

DDA - data-dependent acquisition

DIA - data-independent acquisition

DTT - dithiothreitol

HCD - high energy collision dissociation

HSA - human serum albumin

IAA - 2-iodoacetamide

LC-MS - liquid chromatography-mass spectrometry

LFQ - label-free quantitation

PRM - parallel reaction monitoring

PSM - peptide spectrum matches

QCLMS - quantitative crosslinking/mass spectrometry

SCX - strong cation exchange chromatography

SRM/MRM - selected reaction monitoring/multiple reaction monitoring

URPs - unique residue pairs

* contact: juri.rappsilber@tu-berlin.de

Abstract

Quantitative crosslinking mass spectrometry (QCLMS) reveals structural details of protein conformations in solution. QCLMS can benefit from data-independent acquisition (DIA), which maximises accuracy, reproducibility and throughput of the approach. This DIA-QCLMS protocol comprises of three main sections: sample preparation, spectral library generation and quantitation. The DIA-QCLMS workflow supports isotope-labelling as well as label-free quantitation strategies, uses xiSEARCH for crosslink identification, and xiDIA-Library to create a spectral library for a peptide-centric quantitative approach. We integrated Spectronaut, a leading quantitation software, to analyse DIA data. Spectronaut supports DIA-QCLMS data to quantify crosslinks. It can be used to reveal the structural dynamics of proteins and protein complexes, even against a complex background. In combination with photoactivatable crosslinkers (photo-DIA-QCLMS), the workflow can increase data density and better capture protein dynamics due to short reaction times. Additionally, this can reveal conformational changes caused by environmental influences that would otherwise affect crosslinking itself, such as changing pH conditions.

Introduction

The structure of proteins and protein complexes can be investigated by crosslinking mass spectrometry (CLMS) [1–6] (also abbreviated as CL–MS, XL–MS, CX–MS or CXMS). The approach reveals amino acid residue pairs that are proximal in space by using a crosslinker of known length to form covalent bonds between them. The proteins are then proteolytically digested, crosslinked peptides are detected and crosslinked residue pairs are identified by liquid chromatography-mass spectrometry (LC-MS) paired with database searching.

While a protein's function is linked to its three-dimensional structure, these structures are intrinsically dynamic and can change [7,8]. This also influences the yield of individual crosslinks which is exploited in an approach termed quantitative crosslinking mass spectrometry (QCLMS, also abbreviated as QXL-MS) [9]. When a protein changes its conformation, the distance between its residue pairs may also change, as well as their solvent exposure or orientation towards each other. These changes affect the yield of crosslinks [10]. Detecting changing yields is challenging and requires sensitive instruments in addition to adequate quantitation software as crosslinks tend to be of low abundance in the peptide mixture. Nevertheless, QCLMS is evolving into a complementary tool to traditional structural techniques [3] and has benefited from recent methodological advances. As in linear quantitative proteomics, QCLMS studies comprise two major quantitative strategies: isotope labeling and label-free (LFQ) approaches. To date, QCLMS has been used in a wide range of application cases [11] with isotope label-based strategies used more widely. However, label-free approaches are attracting increased interest. They do not require isotope-labelled crosslinkers, isotope-labelled proteins or isotope-labelling of peptides. Not relying on isotope labels reduces the initial investment in reagents of an experiment. However, conducting the experiment and analysis reproducibly becomes more important.

Currently, QCLMS analysis mainly relies on data-dependent acquisition (DDA). Quantitation in DDA mode is usually performed using extracted ion intensities of the unfragmented peptides (MS1). The first studies that established QCLMS to quantify structural changes used this approach [12,13] and the reproducibility of CLMS was shown to be in line with the general reproducibility of proteomics [14]. However, DDA mode is poorly reproducible for low abundance proteins and their peptides [15–17], which negatively influences quantitation results, especially for the frequently low abundance crosslinks.

Accuracy and reproducibility of QCLMS analysis can be improved by data-independent acquisition (DIA) [18]. DIA is an acquisition method that combines high throughput of DDA with the sensitivity of targeted acquisition methods (selected, parallel or multiple reaction monitoring; SRM, PRM, MRM) [19–21].

DIA workflows in general fall into two basic strategies: peptide-centric [16] and spectrum-centric analysis [22,23]. In both strategies, precursor and fragment quantities are extracted from DIA data using a spectral library and retention time. In peptide-centric workflows, spectral libraries are acquired in DDA mode using either the same sample as for the DIA acquisitions or fractionated samples to increase the size of the library. In spectrum-centric workflows, spectral libraries are generated directly from the DIA data. MS1 and MS2 level information are aligned by retention time and combined in groups to generate pseudo-DDA spectra. These pseudo spectra are used to generate the spectral library for DIA quantitation. Although a spectrum-centric approach offers discovery-like DIA quantitation, it is not yet established for crosslinking data. This protocol therefore focuses on the peptide-centric DIA approach.

Protocol development

General considerations

Here we describe a detailed and automated DIA-QCLMS protocol using a peptide-centric approach. Label-free or labeling strategies can be combined with this workflow. We particularly focus on the DIA method optimisation and software part of the workflow. Sample preparation and enrichment strategies for crosslinking experiments are described in detail by Chen *et al.* 2019 [24]. Our workflow comprises three modules: sample preparation for quantitative crosslinking, spectral library generation and quantitation of crosslinks using DIA (Fig. 1). Note that this protocol uses but is not dependent on xiSEARCH for the identification of crosslinks; other identification software are also compatible.

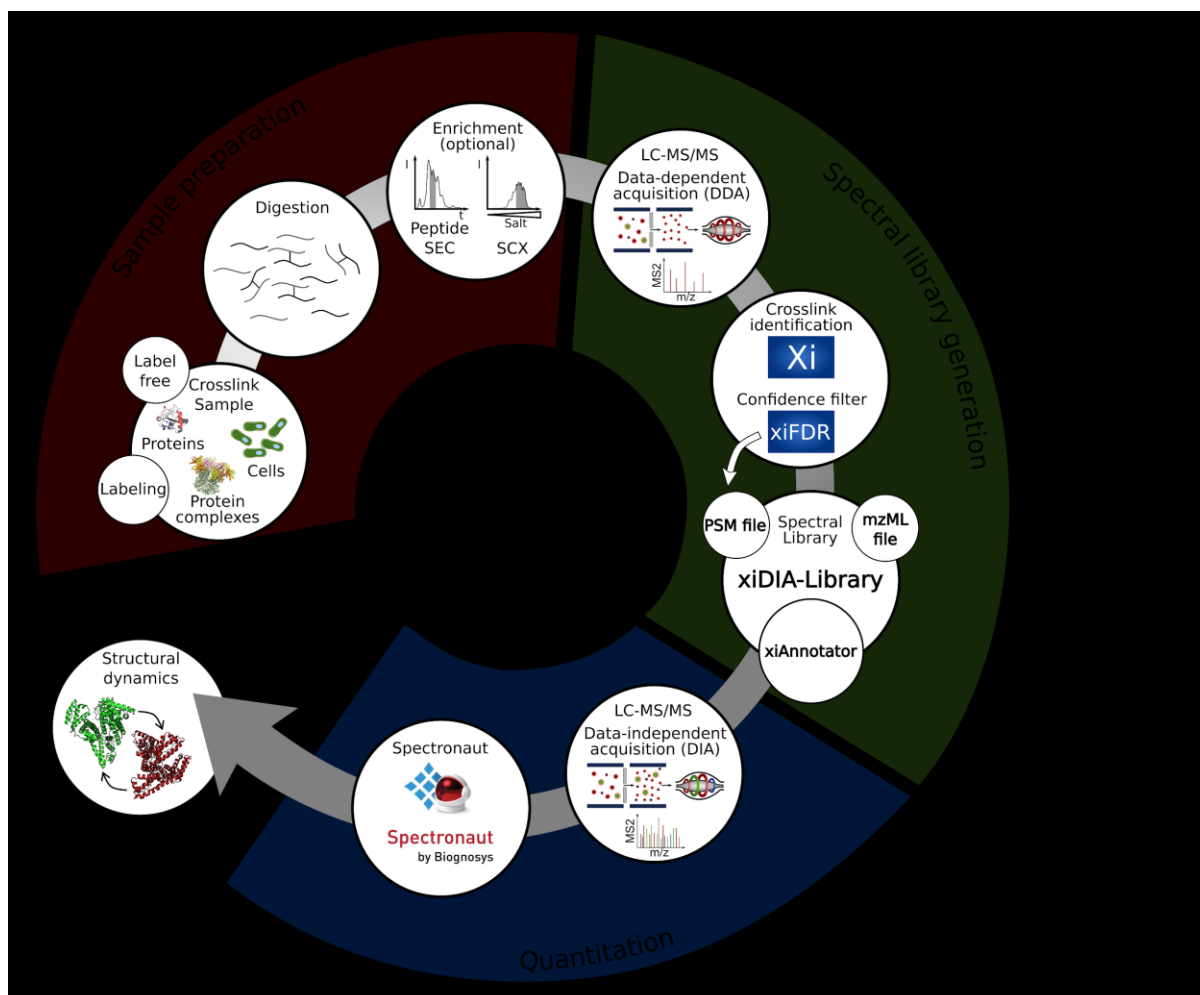


Fig.1: Data-independent quantitative crosslinking mass spectrometry workflow comprising three main modules: sample preparation, spectral library generation and quantitation.

In QCLMS studies, the interpretation of structural changes are based on the signal intensity (peak area) of individual crosslinks (unique residue pairs; URPs). A unique residue pair designates a given combination of two residues that is counted only once, regardless of how many times it was detected as crosslinked in the analysis. Importantly, unique residue pairs are often supported by several different peptide pairs, which are in turn frequently supported by multiple peptide-spectrum matches (PSMs). To obtain the quantitation value for a URP, we take the median signal intensities of all its supporting crosslinked PSMs. In standard quantitative proteomics, this corresponds to combining peptide signals to a protein value [25]. It is worth noting that using a TopN approach for summing up crosslinked peptides to URPs leads to inaccurate quantitative values [18]. It is more accurate to use all supporting crosslinked PSMs for a URP instead of just a subset.

In recent years, significant progress in software development for QCLMS workflows have pushed the field forward [24,26]. Several software packages like xTract [27], MassChroQ [28], pQuant [29], XiQ [12], Skyline [14,30], Pinpoint [10] and MaxQuant [31] support crosslink quantitation on MS1 level in DDA data. Quantitation signals of crosslinked peptides are matched between MS runs through retention time alignment (match between runs), which increases the completeness of quantitative data sets. However, currently only Skyline provides an interface for easy and fast visualisation, and correction of the quantitation results obtained by DDA. There are currently no specialised software tools for analysis of DIA-QCLMS data.

Spectronaut [32,33] a widely used DIA quantitation tool in standard proteomics, was adapted by us to analyse crosslinking data [18]. This software package offers an interface for easy visualisation of DIA crosslinking data, and includes adaptable quantitation setting, statistics and plots to analyse and explore the data. Additionally, no prior programming knowledge is required to use Spectronaut for QCLMS analysis. Detailed manuals and tutorials on the general use of Spectronaut are available on the manufacturer's website (<https://www.biognosys.com/>).

Spectronaut requires a spectral library to extract MS1 and MS2 information from the DIA crosslinking data. This library can be generated from DDA data by our xiDIA-library application [18]. Note that xiDIA-library is an open source collaborative initiative available at its GitHub repository (<https://github.com/Rappsilber-Laboratory/xiDIA-library>). 'Linearisation' of crosslinked peptide sequences as in previous protocols using Skyline is not necessary [14]. xiDIA-library can generate libraries for label-free and labeling experiments. Our library application is written in Python and can be adapted to crosslink identification tools other than xiSEARCH by following the instructions provided at its GitHub repository. Once the spectral library has been imported into Spectronaut, it is used to align retention times using iRT [34] values and extract the MS1 and MS2 information for quantitation.

DIA acquisition

The optimal acquisition parameters of DIA depend on the complexity and dynamic range of the sample. Choosing values for all DIA parameters can be challenging. This is exasperated by the wide range of synonyms and method designs (e.g. WiSIM [35], pSMART [36], HRM [32], SWATH [16], MSX [37], overlapping windows [38]). A very instructive tutorial on general use of DIA in proteomics can be found at Ludwig *et al.* [39]. In this section, we focus on the main features for crosslink DIA optimisation on Orbitrap instruments: the precursor m/z range to cover, number of isolation windows and their widths, injection time and resolution, cycle time and chromatographic peak width (Fig. 2). Starting values for parameter optimisation are given in Tables 1 and 2.

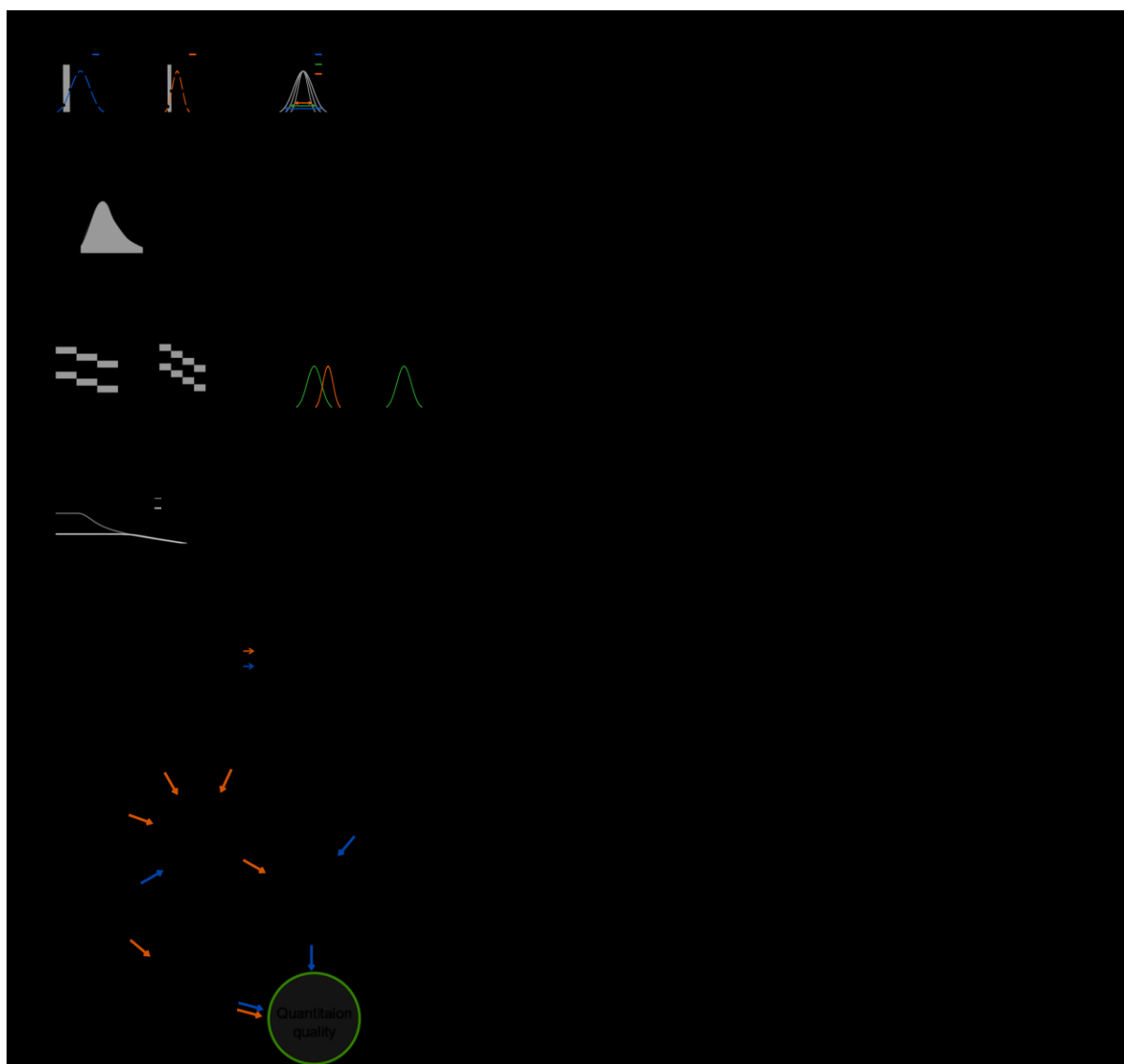


Fig. 2: Optimisation scheme for data-independent acquisition (DIA) methods on Orbitrap instruments. *a:* General guideline and key parameters for DIA optimisation. *b:* Positive and negative dependencies of the key parameters (orange arrow: increasing this parameter will influence the next parameter negatively, blue arrow: increasing this parameter will influence the next parameter positively).

The precursor m/z range covered in a DIA acquisition is defined by consecutive isolation windows with a specific window width. During chromatographic separation, the instrument cycles repeatedly through this set of isolation windows. This mass range is dependent on the sample type as well as the protease used for digestion and should ideally cover the m/z space of crosslinked peptides. Typically, most detected tryptic linear peptides fall within 400-1200 m/z [16], and this generally also applies to crosslinked peptides.

The precursor isolation window width defines a precursor m/z range that will be fragmented for a given MS2 scan. Precursor masses falling within an isolation window are co-isolated and co-fragmented. Hence, the width of a window influences the selectivity, dynamic range and, in turn, also the sensitivity of crosslinked peptide detection. Window width is one of the key parameters during the DIA optimisation process. Narrow isolation windows reduce the number of co-isolated and hence co-fragmented precursors, which results in simpler MS2

spectra and reduced signal interference. However, narrow windows also increase the cycle time and reduce the number of data points per chromatographic peak. In contrast, choosing a wider isolation window allows for faster cycle times, however this increases the number of co-fragmented precursors, resulting in convoluted MS2 spectra and lower sensitivity. Variable window sizes can be applied to balance the intensity distribution of precursor ions across the chromatographic separation and the number of co-fragmented ions. Note that this option is currently not available on all Orbitrap instruments.

The time required to collect ions for the Orbitrap analysis is called 'injection time'. This injection time is determined by the automatic gain control (AGC), which ensures that the mass spectrometer collects the desired number of ions before recording a spectrum. The user-defined 'maximum injection time' limits this time and should be adjusted with respect to the sample complexity. Low sample complexity (e.g. single proteins) often require a higher injection time to fill the trap than complex samples (whole cell lysates).

Together, the injection time, scan time (resolution), and defined window size influence the cycle time. The cycle time (synonyms: duty cycle, sampling rate) refers to the time that is needed to acquire an MS1 spectrum and its subsequent corresponding MS2 spectra. The cycle time determines how often ions of the same peptide are scanned along a chromatographic peak. A short cycle time leads to an increased number of data points per chromatographic peak, which enhances the accuracy of quantitation results. It is recommended to use at least 6 data points per peak for quantitation. Increasing the number of data points per peak also enhances reconstruction of the peak shape [40]. Liquid chromatography performance also influences the number of data points per peak. A cycle time of e.g. 3 seconds is appropriate for an average peak width of 30 seconds, but LC with better resolution reduces the peak width and hence the data points along the chromatographic peak. In this case, the gradient length should be adapted instead.

Table 1: Starting values for MSX-DIA method optimisation.

Parameter	Setting
Isolation window	15
Number of multiplexed ions	2
Collision energy	30 %
Detector type	Orbitrap
Scan range	300-1600
Precursor mass range	400-1200
Max. injection time	90 ms
AGC target	2×10^5
Isolation mode	Quadrupole
Resolution MS2	30000
Resolution MS1	120000

Table 2: Starting values for fixed window DIA method optimization.

Parameter	Setting
Isolation window	12
Number of multiplexed ions	0
Collision energy	30 %
Detector type	Orbitrap
Scan range	300-1600
Precursor mass range	400-1200
Max. injection time	50 ms
AGC target	1x10 ⁵
Isolation mode	Quadrupole
Resolution MS2	30000
Resolution MS1	120000

Applications and limitations

QCLMS data can provide information on protein folding and interactions, and can also reveal regions that exhibit conformational changes. Changes in crosslinked peptide intensities can elucidate protein dynamics [13,26,41–44]. Studies using quantitative crosslinking mass spectrometry (QCLMS) have provided concepts and techniques for studying changing protein states [9] including activation [45], regulation of protein networks [10,41,46,47], maturation of complexes [42], regulation of enzyme activity [13,48,49], protein-protein interactions [50,51] and interactome analysis of cancer cell lines [52]. Quantitative crosslink data have also been applied to support structural modelling and docking experiments to generate high-resolution models of individual protein states [27,43,47]. Particularly for modelling of protein states, it is important to provide accurate and reproducible data while avoiding missing values. DIA-QCLMS improves on DDA based quantitation data [18] and provides higher coverage and fewer missing values [17]. Currently, just a few studies have been published using DIA in conjunction with crosslinking [18,53,54]. DIA-QCLMS is capable of detecting changing abundances of crosslinked peptides, even with the ratio compression encountered with increased sample complexity [18]. In combination with photoactivatable crosslinkers, DIA-QCLMS (photo-DIA-QCLMS) was able to distinguish pH-dependent conformers of Human serum albumin and Cytochrome C [54]. Although DIA-QCLMS has widened the scope of quantitative crosslinking in structural biology, it has been restricted by a lack of software tools supporting DIA crosslink data analysis. This is improving thanks to software development by the fast-growing DIA community.

Materials

To avoid contamination during the sample preparation, it is recommended to work in a laminar flow hood wearing appropriate gloves and lab coat. When using highly sensitive mass spectrometers, sample contaminations increase the dynamic range problem, which can disturb spectral library matching to crosslink DIA data.

Reagents

- PAGE gel (NuPAGE Bis-Tris precast gels; Thermo Fisher Scientific, cat. No. NP0321BOX)
- NativePAGE™ 3-12% Bis-Tris Protein Gels (Thermo Fisher Scientific, cat. No. BN1001BOX)
- NativePAGE™ Cathode Buffer Additive (20X) (Thermo Fisher Scientific, cat. No. BN2002)
- NativePAGE™ Running Buffer (20X) (Thermo Fisher Scientific, cat. No. BN2001)
- NativePAGE™ Sample Buffer (4X) (Thermo Fisher Scientific, cat. No. BN2003)
- NativeMark™ Unstained Protein Standard (Thermo Fisher Scientific, cat. No. LC0725)
- Reagents for SDS gel electrophoresis (LDS sample buffer; MOPS SDS running buffer (20x); MES SDS running buffer (20x); NuPAGE, cat. No. NP0008, NP0001, NP0002, respectively)
- Coomassie staining solution (InstandBlue; Expedeon, cat. No. ISB1L)

Equipment

- Gel electrophoresis chamber (XCell SureLock Mini-Cell electrophoresis system; Thermo Fisher Scientific, cat. No. EI0001)
- Protein LoBind sample tubes (0.5 mL and 1.5 mL; Eppendorf, cat. No. 022431064 and 022431081)
- Thermal mixer for 1.5 mL tubes (Eppendorf, ThermoMixer C model)
- Self-made C18 StageTips [55]
- HPLC column (e.g. EASY-Spray column 50 cm x 75 µm ID, PepMap C₁₈, 2 µm particles, 100 Å pore size, Thermo Fisher Scientific, Germany)
- 96-Well sample plate for LC-MS/MS injections (e.g. PCR microplate, cat. No. 38099 and silicone sealing mat, cat. No. 38107; Axygen Scientific)
- Vacuum centrifuge (e.g. Eppendorf, model No. Concentrator 5301)
- HPLC-mass spectrometer system (e.g. Thermo Fisher Scientific, Ultimate 3500-RS Nano Orbitrap Fusion Lumos Tribrid)
- 3M Empore C18 Extraction Disk (Fisher Scientific, cat. No. 14-386-2)
- Pipettes (0.1-2 µL, 1-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL, Gilson)
- Scarpel (Cutfix disposable scalpels, Carl Roth, art. No. T988.1)

Reagent setup

Crosslink buffer for NHS-ester crosslinkers

The crosslink buffer should preserve the native structure of a target protein or protein complex but also be compatible with the crosslinking reaction. A suitable buffer substance for NHS-

crosslinking is HEPES at a concentration of 20 mM. The pH of the buffer solution should be adjusted to 7.8 using KOH. Salts (e.g. NaCl, Mg₂Cl) and other protein-stabilising reagents such as glycerol (up to 10% v/v) or common additives (10mM DTT, 1mM EDTA) are compatible with the crosslinking reaction. Buffer additives containing e.g. primary amines can react with the crosslinker and must be avoided.

100 mM Ammonium bicarbonate (ABC, NH₄HCO₃)

The 100 mM NH₄CO₃ stock solution needs to be diluted, using bidest water, to 50 mM prior to in-gel digestion. NH₄HCO₃ should be stored at 4°C to avoid decomposition into NH₃, CO₂ and H₂O over time, following an increase in pH.

Destaining solution for in-gel digestion

The destaining solution should always be prepared fresh using MS-grade acetonitrile (ACN), since this solution is used to destain and clean the gel bands prior to trypsin digestion to avoid contamination of the sample. 30% ACN in 50 mM ABC buffer is used for lightly stained protein gel bands. If protein bands are heavily stained, the ACN proportion can be increased up to 50% in ABC buffer combined with heating at 30°C.

Reduction buffer, 10 mM Dithiothreitol (DTT)

The 1 M stock solution needs to be diluted using 50 mM ABC buffer to 10 mM DTT prior to usage. DTT stock solutions are affected by hydrolysis, but can be stored at -20°C in small aliquots for up to six months.

Alkylation buffer, 55 mM Iodoacetamide (IAA)

IAA solutions for in-gel digestion should always be prepared fresh in 50 mM ABC buffer prior to usage. IAA is sensitive to light, therefore solutions should be stored in the dark e.g. wrapped in aluminum foil.

Digestion buffer

The in-gel digestion buffer should be prepared using 50 mM ABC buffer and 5% ACN (v/v). This buffer should always be prepared fresh.

Trypsin stock

Trypsin stock solutions can be prepared using either 0.1% (v/v) TFA or 0.1% (v/v) HCl to avoid self-digestion of the protease, at a concentration of 0.5 µg/µL. The stock can be stored at -80°C in small aliquots (see also specification sheet of the chosen company). Repeated thawing and freezing cycles are not recommended.

10% (v/v) Trifluoroacetic acid (TFA)

The TFA stock solution should be diluted using bidest water to 10% (v/v) TFA. The solution can be stored at room temperature.

0.1% (v/v) Trifluoroacetic acid (TFA)

The 10% TFA solution should be diluted using bidest water to 0.1% (v/v) TFA. The solution can be stored at room temperature.

80% (v/v) Acetonitrile (ACN) in 0.1% (v/v) Trifluoroacetic acid (TFA) (StageTip eluent)

The StageTip eluent solution can be prepared by mixing ACN with 10% (v/v) TFA solution to a final percentage of 80% ACN and 0.1% TFA. The solution can be stored at room temperature.

LC-MS mobile phase A

As mobile phase A, 0.1% formic acid (FA) in water was used. This solution can be stored at room temperature for up to two weeks.

LC-MS mobile phase B

As mobile phase B, 80% (v/v) ACN in 0.1% formic acid (FA) was used. This solution can be stored at room temperature for up to two weeks.

Equipment setup

C18 StageTips

We used self-assembled StageTips for peptide desalting. Further details are described in the protocol by Rappsilber *et al.* [55,56]. In brief, C18 Empore extraction disks were assembled in a 200 μ L pipette tip. The number of extraction disks stacked on top of each other depend on protein amount in the sample. Each extraction disk has an estimated peptide-binding capacity of 15 μ g. Several extraction disks or/and a larger disk diameter can be used to increase peptide loading amount. Prior to peptide loading, C18 StageTips must be freshly activated using methanol and equilibrated with 0.1% (v/v) TFA. The disks must be kept wet during the desalting process in order to prevent peptide loss.

LC-MS

A wide range of HPLC instruments in combination with high resolution reverse-phase columns can be used to study crosslinked peptides. Our DIA-QCLMS protocol followed a standard setup for bottom-up proteomics using an Ultimate 3500-RS Nano LC system coupled with a tribrid Orbitrap mass spectrometer (Orbitrap Fusion™ Lumos, Thermo Fisher Scientific, California, USA). Good resolution and high reproducibility of retention times are especially important for studying crosslinked peptides. Therefore, we applied a commercially available EASY-Spray column (50 cm x 75 μ m ID, PepMap C₁₈, 2 μ m particles, 100 Å pore size, Thermo Fisher Scientific, Germany) using a column temperature of 45-50°C. iRT peptides (Biognosys, Switzerland) were added to each sample before MS acquisition for retention time alignment prior to peptide quantitation. Peptides were separated using a linear 150 min gradient with increasing amounts of ACN. A detailed description of recommended gradients and DDA acquisition strategies for QCLMS can be found in Chen *et al.* 2019 [24]. In short, precursor ions were detected in the Orbitrap at 120,000 resolution using *m/z* range 400-1600. Ions with charge states from 3+ to 7+ were selected for fragmentation. Selected ions were isolated and fragmented by high energy collision dissociation (HCD) and detected in Orbitrap at 30K resolution [57].

DDA acquisition was only used for spectral library generation. For high quality QCLMS analysis, we optimised DIA strategies for a reasonable amount of data points per elution peak (MS1 and MS2) and number of quantified-to-identified crosslinked peptides and intensity of crosslinked features. In our protocol, we focused on fixed window DIA strategies and

multiplexing DIA (MSX). For the fixed window acquisition, precursor ions were acquired using a MS1 master scan (m/z range: 400-1200, max. injection time: 60 ms, AGC target: 4×10^5 , detector: Orbitrap, resolution: up to 120,000 K), followed by 66 DIA scans for MS2 within a fragmentation range of m/z 120-1200 using an isolation window width of m/z 12 and a max. injection time of 50 ms. Selected ions were isolated in the quadrupole, fragmented using HCD (normalised collision energy 30%) and detected in Orbitrap at 30,000 resolution. For the MSX strategy, we acquired two windows (m/z 15) in parallel across a fragmentation range of m/z 120-1200. The injection time was set to 80 ms whereas other MS2 settings were left as for fixed window DIA.

Software

MSconvert, a module of the ProteoWizard Toolkit [58] (<http://proteowizard.sourceforge.net>) was used to process data into peak lists and convert raw files into mgf files.

xiSEARCH [59] (<https://github.com/Rappsilber-Laboratory/xiSEARCH>) was used for identifying crosslinked peptides.

xiFDR [60] (<https://github.com/Rappsilber-Laboratory/xiFDR>) was used for crosslink validation and error estimation.

Preprocessing Python script (<https://github.com/Rappsilber-Laboratory/preprocessing>) was used to convert raw into mgf files and recalibrate precursor masses.

xiAnnotator (<https://github.com/Rappsilber-Laboratory/xiAnnotator>) was used to include fragment annotation into the spectral library.

MaxQuant [25] (<https://www.maxquant.org/>) was used to read out the retention time of spiked in iRT peptides.

xiDIA-library script (<https://github.com/Rappsilber-Laboratory/xiDIA-library>, which is compatible with Python 2.7 or higher) was used to create the spectral library.

Spectronaut v. 11 or 12 (<https://biognosys.com>) was used to for crosslink quantitation.

Python v. 3.6 (<https://www.python.org/>) was used for data processing and visualisation of quantitation results.

PC Hardware

A personal computer with quad-core processor (3.2 GHz), 64-bit Windows system (Win 10) and 8 GB RAM was used to develop this protocol. The minimum requirements for software installations are 4 GB RAM and Windows XP. 4 GB enabled database searches using *xiSEARCH* to study large protein complexes or simple protein mixtures containing up to seven proteins.

Description of procedure

Preparative label-free quantitative crosslinking (sample preparation)

For optimal reactivity of the crosslinker, it is crucial to consider the right protein-to-crosslinker ratio, the composition of crosslinking buffer and pH dependence of the protein/protein complex. Detailed considerations and descriptions are reported by Chen *et al.* 2019 [24]. Ideally, the crosslinking buffer should maintain the native fold of the target protein/protein complex and should not interfere with the crosslink reaction. The final protein concentration of a crosslink reaction should aim for 0.5-1 µg/µL. Additionally, this concentration needs to be in consonance with the critical protein concentration of the target protein to avoid aggregation. The temperature and reaction time during the crosslink reaction should be adjusted to support stability of the target protein/protein complex, but also to minimise the hydrolysis rate of NHS-ester groups of the crosslinker in solution.

For label-free quantitation, a minimum of 10 µg protein is necessary to acquire DIA data in triplicates and construct the spectral library using DDA. If enrichment or fractionation steps are needed, the amount of starting material needs to be adjusted.

- 1) Transfer the protein sample to crosslinking buffer, if not done during the last step of protein purification. Split sample into aliquots if “reaction” replicas are needed (use 15 µg starting material in this case). Adjust the desired temperature prior to the crosslinking reaction (e.g. on ice at 4°C).
- 2) Prepare a fresh stock of crosslinker (e.g. BS³ at 30 µg/µL) in crosslinking buffer.
- 3) Dilute the crosslinker stock into each sample to reach the predetermined optimal protein-to-crosslinker ratio.
- 4) Incubate on ice for one hour and stop the reaction using reagents that interfere with the crosslinker (e.g. 2.5 M ABC solution). Primary amines should be added with a 100-fold excess to the crosslinker in order to enhance efficient quenching of the reaction.
- 5) Incubate 30 min at 4°C.
- 6) Mix the sample with NuPAGE LDS sample buffer (dilute the 4x sample buffer into the sample to reach 1x concentration). Add DTT to a final concentration of 50 mM to the sample and incubate for 5 min at 75-90°C. Note that high temperatures (e.g. 90°C) can result in precipitation of hydrophobic proteins.
- 7) Load the protein samples onto the desired NuPAGE Tris-Bis gel and separate proteins with a constant voltage of 190 V using an appropriate running buffer (e.g. MES or MOPS SDS buffer). Note that lowering the voltage may reduce smearing artifacts, but also reduces the resolution of the separation due to “blurred” protein bands.
- 8) Remove the gel from the electrophoresis tank and wash with bidist water for 5 min on a shaker.
- 9) Stain the gel using coomassie staining (e.g. IstandBlue) until protein bands are visible (approximately 30 min) or use silver staining for low abundance protein bands.
- 10) Destain the gel using water until the protein bands stand out from the background. At this point, crosslinking products can be subjected to in-gel digestion.

Optional: Native-gel electrophoresis for protein complexes > 200 kDa

- A. Mix the sample with NativePAGE™ Sample Buffer (dilute the 4x sample buffer into the sample to reach 1x concentration).
- B. Place the NativePAGE Bis-Tris Gel System into a cool cabinet (~ 4°C).
- C. Load the protein samples onto the desired NativePAGE Bis-Tris gel and apply a voltage of 120 V for about 30 min for gentle migration of the complex into the gel. Separate the proteins using a constant voltage of 160 V for ~ 3h and an appropriate

- anode and cathode buffer (e.g. NativePAGE™ Running Buffer (20x) and NativePAGE™ Cathode Buffer Additive (20X))
- D. Remove the gel from the electrophoresis tank and wash with distilled water for 5 min on a shaker.
 - E. Place gel in fixing solution (40% Methanol, 10% acetic acid) and incubate for 30 min. Stain the gel using coomassie staining (~ 30 min).
 - F. Destain the gel using water or 8% acetic acid until the protein bands stand out from the background. At this point, crosslinking products can be subjected to in-gel digestion.

In-gel digestion of crosslinked protein bands

To minimise peptide loss during digestion, LoBind tubes should be used from step 21 on.

- 11) Excise desired crosslinked protein bands using a scalpel.
- 12) Transfer the complete band into a 1.5 mL reaction tube and wash the gel band by adding distilled water. The volume of liquid should always cover the gel bands. Incubate the sample for 10 min. in a ThermoMixer at RT and 700 rpm. Remove the supernatant after incubation. Repeat this step.
- 13) Add the destaining solution until gel bands are covered with solution and incubate at RT and 700 rpm for 20 min. Discard the supernatant and repeat this step until gel bands are completely destained.
- 14) Add pure ACN until the gel bands are covered and incubate for at least 10 min at RT and 700 rpm. Gel bands will shrink during this process (white colour of the bands indicate successful destaining). Discard the supernatant. Dehydration of the gel bands is important to increase the reaction interface of the gel after rehydration with reduction or alkylation solution. Some protocols even recommend shrinking the gel bands further using a Vacufuge Concentrator.
- 15) Add the same volume of reduction buffer as used during the washing steps. Incubate at 50°C and 700 rpm for 30 min. Incubation with reduction solution will rehydrate the gel pieces by absorbing the solution. Complete hydration will increase the reaction interface and hence enhance the reduction of disulphide bonds of the protein by DTT. Discard the supernatant.
- 16) Add pure ACN to shrink and clean the gel bands prior to the alkylation step. Incubate at RT and 700 rpm for at least 10 min (gel bands need to dry before adding alkylation solution). Discard the supernatant.
- 17) Add the same volume of alkylation buffer as used during the washing steps. Incubate at RT and 700 rpm in the dark (IAA is light sensitive!) for 30 min. This step will alkylate the thiol group of cysteine side chains. Discard the supernatant afterwards.
- 18) Add destaining buffer to wash the gel bands. Incubate at RT (23°C) and 700 rpm for at least 10 min. Discard the supernatant. Cut the gel bands within the reaction tube into small cubes using a scalpel or a small spatula.
- 19) Add pure ACN to shrink the gel bands. Incubate at RT and 700 rpm for at least 10 min. Discard the supernatant.
- 20) Prepare the digestion solution on ice by adding trypsin to the digestion buffer. The amount of trypsin depends on the amount of protein in the sample. A final protease to protein ratio of 1:20 to 1:100 (w/w) is recommended. Incubate at 37°C and 700 rpm overnight (~ 15 h).

- 21) Collect the supernatant after overnight digestion and transfer it to a LoBind tube (extract tube).
- 22) Add 80% (v/v) ACN in 0.1% (v/v) TFA to the gel pieces until they are covered with liquid and incubate for 20-30 min at RT and 700 rpm. This step will increase the extraction yield of peptides through enhanced diffusion from the gel to the supernatant. Additionally, reducing the pH using TFA will stop trypsin digestion as well as self-digestion of the protease to avoid dilution of target peptides with linear tryptic peptides. Collect the supernatant and transfer it to the extraction tube.
- 23) Add pure ACN to shrink the gel pieces completely. Incubate for 10 min at RT and 700 rpm. Collect the supernatant and transfer it to the extraction tube.
- 24) Reduce the final ACN proportion of the extract to less than 5% using a vacuum concentrator to support optimal binding of crosslinked peptides to the C18 StageTip column.
- 25) Add 0.1% (v/v) TFA to adjust pH of the extract to 3 prior to the desalting. For a detailed description of desalting using StageTips, follow the published protocol of Rappsilber *et al.* 2007 [55].

LC-MS/MS analysis

- 26) Elute desalted crosslinked peptides from the C18 StageTip column using 20 μ L StageTip eluent (Reagent setup) into a LoBind tube. Repeat this step once to a final volume of 40 μ L. Dry down the peptides completely using a vacuum concentrator at 45 or 60°C.
- 27) Resuspend crosslinked peptides using 2% (v/v) ACN in 0.1% (v/v) FA. Adjust the final concentration according to your peptide amount to get 0.5-1 μ g peptides per injection.
- 28) Analyse the sample using LC-MS/MS as described in the equipment setup section. 2 μ L (1 μ g) of peptides is injected for each DDA and DIA acquisition. The injection volume can be adjusted according to individual LC setups.

Crosslinked peptide identification and spectral library generation

Use the whole set of DDA acquisitions, including all replica, for crosslinked peptide identification. In general, any available crosslink identification tool can be used for spectral library generation. Note that this protocol focuses on xiSEARCH and provides a detailed description on how to setup the pipeline using tools from the Rappsilber laboratory.

- 29) Optional: download the preprocessing Python script from GitHub (see Software section) and follow the setup instructions for the preprocessing tool.
- 30) Copy your raw files into the folder called “rawfiles” and insert your desired fasta file into the global folder. Rename your fasta file to “DATABASE” in order to use the original batch file (“command_dev”). If you don’t want to rename your fasta file, change the name in “command_dev” by opening the batch file using an editor tool. If the target sample contains several proteins, combine all fasta files into a single file.
- 31) Execute the “command_dev” file to start the preprocessing. Results will be collected in the “processed” folder.
- 32) Steps 29-31 are optional and can be replaced by simply opening MSConvert and converting raw files to mgf. Select “Peak Picking” in filter options in profile mode during MS2 acquisition.
- 33) Set up xiSEARCH on a PC as described on GitHub or in Chen *et al.* 2019 [24].

- 34) Open xiSEARCH, go to the “Peak Lists” tab, click the select button and select all recalibrated or non-recalibrated mgf files.
- 35) Go to the “Fasta Files” tab and upload the desired fasta file.
- 36) All search parameters can be set up in the “Config” tab. Search for the parameters you want to change by pressing Ctrl+F. The Config is set up by default for BS³ but contains descriptions on how to set up other crosslinkers. Detailed parameters are listed in Table 3.
- 37) Define the directory for results in the “Run” tab and press “Start”. Results are saved as a .csv file.

Table 3: Parameters for database search for crosslink identification using XiSearch.

Parameter	Settings
Digest	Trypsin\P
missedcleavages:	3
MINIMUM_PEPTIDE_LENGTH:	6
tolerance:precursor:	6 ppm (adjust according to instrument performance)
tolerance:fragment:	20 ppm (adjust according to instrument performance)
crosslinker:	BS ³
modification:fixed:	Carbamidomethyl (C)
modification:variable:	Oxidation (M), crosslinker modifications
fragment:	b-, y-, precursor-ions
loss:	-CH ₃ SOH, -H ₂ O, -NH ₃
missing_isotope_peaks:	3 (adjust according to instrument performance)

- 38) Open xiFDR [60] and navigate to the “CSV” tab within the “Input” tab. Import the result file from xiSEARCH by pressing the “...” button.
- 39) Navigate to the “FDR Settings” tab and choose the desired FDR settings. Detailed settings can be changed by selecting “complete FDR”. A 1% or 5% link level FDR is commonly used. Start FDR calculations by pressing “Calculate”.
- 40) A summary of the results is displayed in the “Result” tab. Export the results by using the “CSV/TSV” tab, selecting an output path, a name for the file and clicking the “Write” button. It is also recommended to convert the resulting file to mzIdentML for publication. Results can be visualised and explored using xiVIEW by uploading the resulting .csv file to https://xiview.org/xiNET_website/index.php. Follow the instructions on the website to ensure correct importing.
- 41) Download the xiDIA-library tool and follow the instructions on GitHub for installation and setup. Open the Python script called “create_spectronaut_lib.py” and “config.py”.

Modify the config file according to the GitHub instructions and save it. If a labeled experiment was performed, set “label_experiment” to True and specify the labeled settings.

- 42) Select the “*_PSM_xiFDR*” file from your xiFDR results and copy it to the directory specified under “psm_csv_path = baseDir + “psm_csv/””.
- 43) Convert all DDA raw files, used in xiSEARCH, to mzML files and copy the files to the directory specified under “mzml_path = baseDir + “mzml/””.
- 44) In order to calculate iRT values for each identified crosslinked peptide, determine the retention time of iRT peptides within DDA runs. Perform a linear search using MaxQuant (default setting) and the iRT fasta file from Biognosys. Skyline can be also used to obtain the retention times from iRT peptides in DDA runs. Plot the retention time of iRT peptides (y-axis) against the iRT values (x-axis) (obtained from the Biognosys website) and perform a linear fit of the curve. Insert the slope and y-intercept value into the config file (slope = iRT_m, interception = iRT_t).
- 45) If all settings in the config file are specified, press the “Run file” button to start library generation. The spectral library file will be saved in the defined output folder.

MS1 and MS2-based quantitation of DIA-QCLMS data using Spectronaut

The DIA-QCLMS workflow is based on a peptide-centric approach, which uses a spectral library to extract MS1 and MS2 information from DIA data. To align different LC-gradients iRT peptides are spiked into each sample and used for retention time alignment.

- 46) Open the HTRMS converter provided by the Spectronaut software package. Click “Add Files” to import DIA files for converting. Press OK to start the process.
- 47) Open Spectronaut and set up all modifications that were used during xiSEARCH. To do this, navigate to the “Modifications” tab within the “Databases” tab. Click “New” in the lower left corner to open a new modification entry. Provide a name for the modification, specify the composition, modification site and special ions (if necessary). Click “save” or “save as” to save the new entry.
- 48) Navigate to the “Analysis” tab and start the wizard by clicking “Set up a DIA Analysis from File”. Select the HTRMS files that were generated in step 1 and click open. Navigate to “Assign Spectral Library” following “from File” to import the external spectral library created by xiDIA-Library. Select “Browse” and search for the desired .csv file. The library will be displayed within the wizard as a table. Navigate to the column named “cl_residue_pair” and choose “PrecursorComment” from the dropdown menu. This step ensures that crosslinked peptides will be assigned to unique residue pairs (unique links) in the export table after quantitation. Click “Load”. A new window will open to assign synonyms to modifications. Modifications with a red “x” can be assigned by dragging the right modification name to the entry. Click “Apply” to finish this step. The library will now be associated with the DIA files.
- 49) Click “Next” and choose the quantitation settings for your analysis. Example parameters for a DIA-QCLMS analysis are shown in Table 4. Note that if decoys are not provided in the library, tick “Generate Decoys” in the Identification tab.

Table 4: Example settings for DIA-QCLMS in the Quantification tab.

Parameter	Setting
-----------	---------

Major (Protein) Grouping	by Protein Group Id
Minor (Peptide) Grouping	by Modified Sequence
Major Group Quantity	unticked
Minor Group Quantity	Mean precursor quantity
Minor Group Top N	ticked (Max: 10, Min: 1)
Quantity MS-Level	MS2 (or MS1)
Quantity Type	Area
Data Filtering	Qvalue
Normalization Strategy	Local Normalization

- 50) Click “Next”, skip the fasta file selection if no background library (linear peptides) are used in the analysis, and set up conditions of the samples in the following tab. Skip the gene annotation tab and check the analysis setup in the summary tab before clicking “Finish”. Spectronaut will start extracting peptide information and iRT calibration after clicking the “Finish” button.
- 51) After data extraction, select the “Analysis” tab if not done automatically. Click the right mouse button within the window showing the DIA run names to open additional options. Navigate to the “Group By” option and select “Protein Group Id”. Crosslinked peptides are now grouped according to the “ProteinId” column in the library and are displayed in the dropdown menu when clicking the arrow beside the run and protein name.
- 52) Choose “Qvalue”, “Condition CVs” or other options in the dropdown menu in the lower left corner of the window to filter and explore the data.
- 53) Click right in the window to open options and save the analysis.
- 54) The “Post Analysis” tab will give an overview of some general features and results of the analysis. Be aware that not all plots are entirely suitable to represent crosslinking data.
- 55) Navigate to the “Report” tab to export the data for further processing. Include the “FG.Comment” column in the export scheme to show the residue pairs for each crosslinked peptide.

Transition from quantified unique crosslinked peptides to residue pairs

- 56) Open the quantitation report file in either a spreadsheet application or Python, and calculate the median of normalised MS1 and/or MS2 signals of all crosslinked peptides (column: “EG_ModifiedSequence”) that support one crosslinked residue pair (column: “FG.Comment”). Note: if replica were acquired, calculate the median between replica for each crosslinked peptide first, followed by the median per residue pair and condition.
- 57) The final table should now contain one value for each residue pair and each condition

Processing crosslink quantitation results

Quantification data from crosslinked peptides needs to be consolidated into crosslinked residue pairs. It has been shown that alternative proteolytic cleavages and post-crosslinking modifications may lead to variations in quantified signal [10]. Therefore, the peak area corresponding to a crosslinked residue pair is calculated as the median value of the peak areas of all its supporting crosslinked peptide pairs. Contrary to standard proteomics, it is beneficial to use all supporting peptides instead of just Top N to calculate the residue pair signal [18] and reduce the impact of outliers. However, compared to proteins, crosslinked residues are usually supported by fewer peptide features. Therefore, a high reproducibility between replicates is needed when quantifying using isotope labelling or label-free quantitation. A low coefficient of variation (CV) for replicates implies a reliable quantitation results.

Changes in relative peptide abundance can not only represent actual biological changes but also changes as a function of bias and noise. Both bias and noise can lead to variability among replica and can affect accuracy and precision of biological conclusions. Hence, normalisation of the quantitation data is required to account for variabilities. In Spectronaut, two normalisation strategies are available: Central Tendency Normalization (Global Normalization) and Local Regression Normalization (Local Normalization). Central Tendency Normalization centres peptide abundance ratios around a median, mean or a constant to adjust for the effects of independent systematic bias [61]. Local Regression Normalization on the other hand, assumes that systematic bias correlates nonlinearly to peptide abundance. This nonlinear correlation could result from ion suppression on measured abundances, with abundances reaching the detector limit or background (small S/N ratio) [61]. After normalisation of crosslinked peptides and calculation of the median value for each unique residue pair, statistical analyses are necessary to extract significant changes in crosslink abundances over several conditions (protein complex conformations). The ANOVA test can be used to analyse differences between the means of unique residue pairs from different conditions. Significantly changed residue pairs can be displayed in structures or used for modeling purposes. There are different ways to display significantly changing residue pairs. To compare two samples or conditions, a volcano plot is an appropriate way to visualise significant residue pairs (Fig. 3 b). If there are more than two conditions, a cluster plot or heatmap enhances the visualisation of results (Fig. 3 c). Finally, changing residue pairs can be displayed directly in pdb structures to point to regions of interest within a protein (Fig. 3 a). In this protocol, we also provide example raw files, library and result files that can be used to follow the protocol and compare the analysis. Using the provided files, the protocol can be started either from the raw files by performing a xiSEARCH for crosslinked peptide identification (step 29) or from the library file (HSA_sulfoSDA_xiDIA_library_file.csv) to follow the quantitation part (step 46). The “HSA_sulfoSDA_PSMfile_xiFDR1.0.22.46.csv” file can be used to follow the protocol after crosslinked peptide identification (step 41 onwards). Results after quantitation analysis in Spectronaut are collected in the “HSA_sulfoSDA_Spectronaut_Report.xls” file. Note that the results could slightly change when using different versions of Spectronaut (check also release notes).

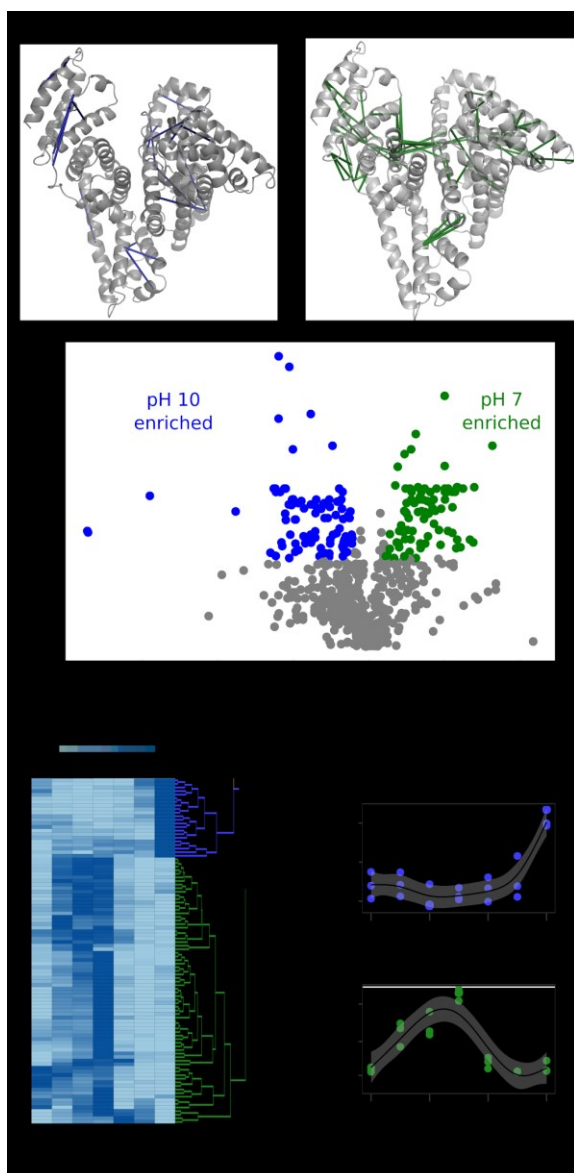


Fig. 3: Visualisation examples of significantly changing residue pairs. *a:* Residue pairs with maximum abundance at pH 7 (green) mapped on the structure of human serum albumin (PDB accession code 1AO6). *b:* Residue pairs with maximum abundance at pH 10 (blue) mapped on the structure of human serum albumin (PDB accession code 1AO6). *c:* Volcano plot after performing a two sided t-test of triplicate datasets of pH 7 and pH 10 using p-value cutoff of 0.05 and fold change cutoff of 1 (blue: significantly changing unique residue pairs for pH 10, green: significantly changing residue pairs for pH 7, grey: residue pairs that have no significant change). *d:* cluster plot adapted and modified from Müller et al. 2019 [54] showing median abundances of URPs and statistically significant shifts as a function of pH ($p < 0.05$) (red: pH 7, green: pH 10).

Concluding remarks

Our DIA-QCLMS workflow ensures high accuracy and precision of quantitation results compared to previous workflows. Particularly low coefficients of variation of peak areas suggest that even small changes in protein states could be detected by QCLMS [18]. Structural changes in proteins can now be monitored across a wide range of environmental changes, including pH [54] but presumably also temperature, pressure or concentration. The

DIA-QCLMS workflow widens the scope of crosslinking applications and makes the analysis of protein complex topologies or protein networks in cellular systems possible.

Acknowledgments

This work was supported by the Wellcome Trust (103139, 108504), an Einstein Visiting Fellowship and the DFG (RA 2365/4-1). The Wellcome Centre for Cell Biology is supported by core funding from the Wellcome Trust (203149).

Data Availability

The mass spectrometry raw files, xiDIA-library and Spectronaut result files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014674.

Reviewer account details:

Username: reviewer87772@ebi.ac.uk

Password: aOkSVC8M

Compliance with ethical standards

Competing interests

The authors declare no financial interest.

References

- [1] A. Sinz, The advancement of chemical cross-linking and mass spectrometry for structural proteomics: from single proteins to protein interaction networks, *Expert Rev. Proteomics*. 11 (2014) 733–743.
- [2] F. Liu, A.J.R. Heck, Interrogating the architecture of protein assemblies and protein interaction networks by cross-linking mass spectrometry, *Curr. Opin. Struct. Biol.* 35 (2015) 100–108.
- [3] A. Leitner, M. Faini, F. Stengel, R. Aebersold, Crosslinking and Mass Spectrometry: An Integrated Technology to Understand the Structure and Function of Molecular Machines, *Trends Biochem. Sci.* 41 (2016) 20–32.
- [4] M. Schneider, A. Belsom, J. Rappsilber, Protein Tertiary Structure by Crosslinking/Mass Spectrometry, *Trends Biochem. Sci.* 43 (2018) 157–169.
- [5] J.D. Chavez, J.E. Bruce, Chemical cross-linking with mass spectrometry: a tool for systems structural biology, *Curr. Opin. Chem. Biol.* 48 (2018) 8–18.
- [6] F.J. O'Reilly, J. Rappsilber, Cross-linking mass spectrometry: methods and applications in structural, molecular and systems biology, *Nat. Struct. Mol. Biol.* 25 (2018) 1000–1008.
- [7] P.G. Debrunner, H. Frauenfelder, Dynamics of Proteins, *Annu. Rev. Phys. Chem.* 33 (1982) 283–299.
- [8] M. Karplus, Dynamics of proteins, *Adv. Biophys.* 18 (1984) 165–190.
- [9] Z.A. Chen, J. Rappsilber, Protein Dynamics in Solution by Quantitative Crosslinking/Mass Spectrometry, *Trends Biochem. Sci.* 43 (2018) 908–920.
- [10] Z. Chen, L. Fischer, S. Tahir, J.-C. Bukowski-Wills, P. Barlow, J. Rappsilber, Quantitative cross-linking/mass spectrometry reveals subtle protein conformational changes, *Wellcome Open Res.* 1 (2016) 5.
- [11] Z.A. Chen, J. Rappsilber, Protein dynamics in solution by quantitative cross-linking/mass spectrometry, *Trends Biochem. Sci.* (2018) in press.
- [12] L. Fischer, Z.A. Chen, J. Rappsilber, Quantitative cross-linking/mass spectrometry using

- isotope-labelled cross-linkers, *J. Proteomics*. 88 (2013) 120–128.
- [13] C. Schmidt, M. Zhou, H. Marriott, N. Morgner, A. Politis, C.V. Robinson, Comparative cross-linking and mass spectrometry of an intact F-type ATPase suggest a role for phosphorylation, *Nat. Commun.* 4 (2013) 1985.
 - [14] F. Müller, L. Fischer, Z.A. Chen, T. Auchynnikava, J. Rappsilber, On the Reproducibility of Label-Free Quantitative Cross-Linking/Mass Spectrometry, *J. Am. Soc. Mass Spectrom.* 29 (2018) 405–412.
 - [15] A. Bensimon, A.J.R. Heck, R. Aebersold, Mass spectrometry-based proteomics and network biology, *Annu. Rev. Biochem.* 81 (2012) 379–405.
 - [16] L.C. Gillet, P. Navarro, S. Tate, H. Röst, N. Selevsek, L. Reiter, R. Bonner, R. Aebersold, Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis, *Mol. Cell. Proteomics*. 11 (2012) O111.016717.
 - [17] A. Hu, W.S. Noble, A. Wolf-Yadlin, Technical advances in proteomics: new developments in data-independent acquisition, *F1000Res*. 5 (2016). <https://doi.org/10.12688/f1000research.7042.1>.
 - [18] F. Müller, L. Kolbowski, O.M. Bernhardt, L. Reiter, J. Rappsilber, Data-independent Acquisition Improves Quantitative Cross-linking Mass Spectrometry, *Mol. Cell. Proteomics*. 18 (2019) 786–795.
 - [19] J.D. Venable, M.-Q. Dong, J. Wohlschlegel, A. Dillin, J.R. Yates, Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra, *Nat. Methods*. 1 (2004) 39–45.
 - [20] A. Bilbao, E. Varesio, J. Luban, C. Strambio-De-Castillia, G. Hopfgartner, M. Müller, F. Lisacek, Processing strategies and software solutions for data-independent acquisition in mass spectrometry, *Proteomics*. 15 (2015) 964–980.
 - [21] R. Bruderer, J. Sondermann, C.-C. Tsou, A. Barrantes-Freer, C. Stadelmann, A.I. Nesvizhskii, M. Schmidt, L. Reiter, D. Gomez-Varela, New targeted approaches for the quantification of data-independent acquisition mass spectrometry, *Proteomics*. (2017) 1700021.
 - [22] C.-C. Tsou, D. Avtonomov, B. Larsen, M. Tucholska, H. Choi, A.-C. Gingras, A.I. Nesvizhskii, DIA-Umpire: comprehensive computational framework for data-independent acquisition proteomics, *Nat. Methods*. 12 (2015) 258–64, 7 p following 264.
 - [23] J. Wang, M. Tucholska, J.D.R. Knight, J.-P. Lambert, S. Tate, B. Larsen, A.-C. Gingras, N. Bandeira, MSPLIT-DIA : sensitive peptide identification for data-independent acquisition, *Nature Publishing Group*. (2015). <https://doi.org/10.1038/nmeth.3655>.
 - [24] Z.A. Chen, J. Rappsilber, Quantitative cross-linking/mass spectrometry to elucidate structural changes in proteins and their complexes, *Nat. Protoc.* 14 (2019) 171–201.
 - [25] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, *Nat. Biotechnol.* 26 (2008) 1367–1372.
 - [26] C. Yu, L. Huang, Cross-Linking Mass Spectrometry: An Emerging Technology for Interactomics and Structural Biology, *Anal. Chem.* 90 (2018) 144–165.
 - [27] T. Walzthoeni, L.A. Joachimiak, G. Rosenberger, H.L. Röst, L. Malmström, A. Leitner, J. Frydman, R. Aebersold, xTract: software for characterizing conformational changes of protein complexes by quantitative cross-linking mass spectrometry, *Nat. Methods*. 12 (2015) 1185–1190.
 - [28] B. Valot, O. Langella, E. Nano, M. Zivy, MassChroQ: a versatile tool for mass spectrometry quantification, *Proteomics*. 11 (2011) 3572–3577.
 - [29] C. Liu, C.-Q. Song, Z.-F. Yuan, Y. Fu, H. Chi, L.-H. Wang, S.-B. Fan, K. Zhang, W.-F. Zeng, S.-M. He, M.-Q. Dong, R.-X. Sun, pQuant improves quantitation by keeping out interfering signals and evaluating the accuracy of calculated ratios, *Anal. Chem.* 86 (2014) 5286–5294.
 - [30] B. MacLean, D.M. Tomazela, N. Shulman, M. Chambers, G.L. Finney, B. Frewen, R. Kern, D.L. Tabb, D.C. Liebler, M.J. MacCoss, Skyline: An open source document editor for creating and analyzing targeted proteomics experiments, *Bioinformatics*. 26 (2010)

- 966–968.
- [31] Z.A. Chen, L. Fischer, J. Cox, J. Rappsilber, Quantitative Cross-linking/Mass Spectrometry Using Isotope-labeled Cross-linkers and MaxQuant, *Mol. Cell. Proteomics*. 15 (2016) 2769–2778.
 - [32] R. Bruderer, O.M. Bernhardt, T. Gandhi, S.M. Miladinović, L.-Y. Cheng, S. Messner, T. Ehrenberger, V. Zanotelli, Y. Butscheid, C. Escher, O. Vitek, O. Rinner, L. Reiter, Extending the Limits of Quantitative Proteome Profiling with Data-Independent Acquisition and Application to Acetaminophen-Treated Three-Dimensional Liver Microtissues, *Mol. Cell. Proteomics*. 14 (2015) 1400–1410.
 - [33] R. Bruderer, O.M. Bernhardt, T. Gandhi, Y. Xuan, J. Sondermann, M. Schmidt, D. Gomez-Varela, L. Reiter, Optimization of Experimental Parameters in Data-Independent Mass Spectrometry Significantly Increases Depth and Reproducibility of Results, *Mol. Cell. Proteomics*. 16 (2017) 2296–2309.
 - [34] C. Escher, L. Reiter, B. MacLean, R. Ossola, F. Herzog, J. Chilton, M.J. MacCoss, O. Rinner, Using iRT, a normalized retention time for more targeted measurement of peptides, *Proteomics*. 12 (2012) 1111–1121.
 - [35] R. Kiyonami, M. Senko, V. Zabrouskov, A.F.R. Hühmer, T.F. Scientific, S. Jose, Large-Scale Targeted Protein Quantification Using WiSIM-DIA on an Orbitrap Fusion Tribrid Mass Spectrometer, (2014) 1–8.
 - [36] A. Prakash, S. Peterman, S. Ahmad, D. Sarracino, B. Frewen, M. Vogelsang, G. Byram, B. Krastins, G. Vadali, M. Lopez, Hybrid Data Acquisition and Processing Strategies with Increased Throughput and Selectivity: pSMART Analysis for Global Qualitative and Quantitative Analysis, *J. Proteome Res.* 13 (2014) 5415–5430.
 - [37] J.D. Egertson, A. Kuehn, G.E. Merrihew, N.W. Bateman, B.X. MacLean, Y.S. Ting, J.D. Canterbury, D.M. Marsh, M. Kellmann, V. Zabrouskov, C.C. Wu, M.J. MacCoss, Multiplexed MS/MS for improved data-independent acquisition, *Nat. Methods*. 10 (2013) 744–746.
 - [38] D. Amodei, J. Egertson, B.X. MacLean, R. Johnson, G.E. Merrihew, A. Keller, D. Marsh, O. Vitek, P. Mallick, M.J. MacCoss, Improving Precursor Selectivity in Data-Independent Acquisition Using Overlapping Windows, *J. Am. Soc. Mass Spectrom.* 30 (2019) 669–684.
 - [39] C. Ludwig, L. Gillet, G. Rosenberger, S. Amon, B.C. Collins, R. Aebersold, Data-independent acquisition- based SWATH- MS for quantitative proteomics: a tutorial, *Mol. Syst. Biol.* 14 (2018) e8126.
 - [40] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, *Mol. Syst. Biol.* 4 (2008) 222.
 - [41] A.P. Herbert, E. Makou, Z.A. Chen, H. Kerr, A. Richards, J. Rappsilber, P.N. Barlow, Complement Evasion Mediated by Enhancement of Captured Factor H: Implications for Protection of Self-Surfaces from Complement, *J. Immunol.* 195 (2015) 4986–4998.
 - [42] R.J. Tomko, D.W. Taylor, Z.A. Chen, H.W. Wang, J. Rappsilber, M. Hochstrasser, A Single α Helix Drives Extensive Remodeling of the Proteasome Lid and Completion of Regulatory Particle Assembly, *Cell*. 163 (2015) 432–444.
 - [43] J.D. Chavez, D.K. Schweppe, J.K. Eng, J.E. Bruce, In Vivo Conformational Dynamics of Hsp90 and Its Interactors, *Cell Chem Biol.* 23 (2016) 716–726.
 - [44] X. Wang, I.E. Chemmama, C. Yu, A. Huszagh, Y. Xu, R. Viner, S.A. Block, P. Cimermancic, S.D. Rychnovsky, Y. Ye, A. Sali, L. Huang, The proteasome-interacting Ecm29 protein disassembles the 26S proteasome in response to oxidative stress, *J. Biol. Chem.* 292 (2017) 16310–16320.
 - [45] B.X. Huang, Interdomain Conformational Changes in Akt Activation Revealed by Chemical Cross-linking and Tandem Mass Spectrometry, *Mol. Cell. Proteomics*. 5 (2006) 1045–1053.
 - [46] B.X. Huang, H.-Y. Kim, Probing Akt-inhibitor interaction by chemical cross-linking and mass spectrometry, *J. Am. Soc. Mass Spectrom.* 20 (2009) 1504–1513.
 - [47] Z.A. Chen, R. Pellarin, L. Fischer, A. Sali, M. Nilges, P.N. Barlow, J. Rappsilber, Structure of Complement C3(H2O) Revealed By Quantitative Cross-Linking/Mass

- Spectrometry And Modeling, *Mol. Cell. Proteomics*. 15 (2016) 2730–2743.
- [48] C. Yu, H. Mao, E.J. Novitsky, X. Tang, S.D. Rychnovsky, N. Zheng, L. Huang, Gln40 deamidation blocks structural reconfiguration and activation of SCF ubiquitin ligase complex by Nedd8, *Nat. Commun.* 6 (2015) 10053.
 - [49] S.G. Boelt, C. Norn, M.I. Rasmussen, I. André, E. Čiplys, R. Slibinskas, G. Houen, P. Højrup, Mapping the Ca(2+) induced structural change in calreticulin, *J. Proteomics*. 142 (2016) 138–148.
 - [50] V. Beilsten-Edmands, Y. Gordiyenko, J.C. Kung, S. Mohammed, C. Schmidt, C.V. Robinson, eIF2 interactions with initiator tRNA and eIF2B are regulated by post-translational modifications and conformational dynamics, *Cell Discov.* 1 (2015) 15020.
 - [51] C. Koehler, P.F. Sauter, M. Wawryszyn, G.E. Girona, K. Gupta, J.J.M. Landry, M.H.-Y. Fritz, K. Radic, J.-E. Hoffmann, Z.A. Chen, J. Zou, P.S. Tan, B. Galik, S. Junttila, P. Stolt-Bergner, G. Pruneri, A. Gyenesei, C. Schultz, M.B. Biskup, H. Besir, V. Benes, J. Rappsilber, M. Jechlinger, J.O. Korbel, I. Berger, S. Braese, E.A. Lemke, Genetic code expansion for multiprotein complex engineering, *Nat. Methods*. 13 (2016) 997–1000.
 - [52] J.D. Chavez, D.K. Schweppe, J.K. Eng, C. Zheng, A. Taipale, Y. Zhang, K. Takara, J.E. Bruce, Quantitative interactome analysis reveals a chemoresistant edgotype, *Nat. Commun.* 6 (2015) 7928.
 - [53] S. Hauri, H. Khakzad, L. Happonen, J. Teلمان, J. Malmström, L. Malmström, Rapid determination of quaternary protein structures in complex biological samples, *Nat. Commun.* 10 (2019) 192.
 - [54] F. Müller, A. Graziadei, J. Rappsilber, Quantitative photo-crosslinking mass spectrometry reveals protein structure response to environmental changes, *Anal. Chem.* (2019). <https://doi.org/10.1021/acs.analchem.9b01339>.
 - [55] J. Rappsilber, M. Mann, Y. Ishihama, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, *Nat. Protoc.* 2 (2007) 1896–1906.
 - [56] J. Rappsilber, Y. Ishihama, M. Mann, Stop And Go Extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics, *Anal. Chem.* 75 (2003) 663–670.
 - [57] L. Kolbowski, M.L. Mendes, J. Rappsilber, Optimizing the Parameters Governing the Fragmentation of Cross-Linked Peptides in a Tribrid Mass Spectrometer, *Anal. Chem.* 89 (2017) 5311–5318.
 - [58] J.D. Holman, D.L. Tabb, P. Mallick, Employing ProteoWizard to convert raw mass spectrometry data, *Curr. Protoc. Bioinformatics*. (2014) 1–9.
 - [59] M.L. Mendes, L. Fischer, Z.A. Chen, M. Barbon, F.J. O'Reilly, M. Bohlke-Schneider, A. Belsom, T. Dau, C.W. Combe, M. Graham, M.R. Eisele, W. Baumeister, C. Speck, J. Rappsilber, An integrated workflow for cross-linking/mass spectrometry, *bioRxiv*. (2018) 355396.
 - [60] L. Fischer, J. Rappsilber, Quirks of Error Estimation in Cross-Linking/Mass Spectrometry, *Anal. Chem.* 89 (2017) 3829–3833.
 - [61] S.J. Callister, R.C. Barry, J.N. Adkins, E.T. Johnson, W.-J. Qian, B.-J.M. Webb-Robertson, R.D. Smith, M.S. Lipton, Normalization approaches for removing systematic biases associated with mass spectrometry and label-free proteomics, *J. Proteome Res.* 5 (2006) 277–286.